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Interfacial Properties of Oligo (ethylene glycol)-Terminated Self-Assembled Monolayers on Stainless Steel 316L

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Interfacial Properties of Oligo(ethylene glycol)-Terminated Self-Assembled Monolayers on Stainless Steel 316L

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Abstract

The implantation of SS316L stento in an artery is associated with a number of problems, including restenosis and thrombosis, due to non-specific protein and cell attachment to the biomaterial. One way to reduce non-specific attachment is to alter the interfacial properties of these materials. This may be done using self-assembled monolayers. Model gold surfaces have been extensively studied and oligo(ethylene glycol) monolayers are the standard for protein and cell mitigation. In this study, self-assembled monolayers were formed on the native oxide surface of stainless steel 316L using synthesized and commercially available phosphonic and carboxylic acids terminated with methyl and triethylene glycol moieties to investigate their ability to mitigate non-specific attachment of 3T3 Swiss fibroblast cells over a twenty four hour time period. Results indicate that methyl-terminated phosphonic acids provided the greatest mitigation of cells which is analogous to two studies on model gold substrates but not to most studies comparing the two modifications.

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Chapter 1: Introduction and Objectives

1.1 Introduction

Biomaterials are artificial materials which are placed inside of a living system and must function intimately with living tissue.[1, 2] They may be used to replace part of a living system, i.e. knee prosthesis, or assist in everyday bodily functions, i.e. stents, in order to assure the natural flow of the body.

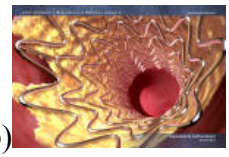
a)  b) 

Figure 1: Examples of a(n) a) Artificial knee implant and b) Stent

Biomaterials can be made from a variety of materials including polymers and metal oxides. The materials are placed inside of the body where they come into contact with bodily fluids that contain proteins and cells.[3] There are severe complications associated with the implantation of biomaterials including corrosion of the material, non-specific protein and cell attachment, and infection at the site of the implant.[1-4]

Due to the intimate contact with bodily fluids, the material must possess the ability to resist corrosion, making titanium, iron, and nickel-based alloys popular metal oxide materials. There is a protective oxide layer covering the metals which enhance corrosion resistant properties of the metals (Figure 2).[2]

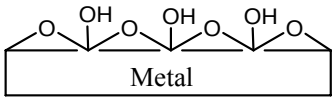


Figure 2: Metal oxides consist of a thin layer of μ-oxo and hydroxyl groups attached to the surface.

In the case of stainless steel implants, low carbon steels such as stainless steel 316L (SS316L) are used because of the trace amounts of carbon present in the alloy. SS316L is composed primarily of iron (~68%), chromium (16-18%), nickel (10-14%), and molybdenum (2-3%), along with trace amounts of carbon, manganese, phosphorus, sulfur, and silicon.[2-4] Trace amounts of carbon in the material makes it less susceptible to corrosion.[5] Carbon increases the corrosion rate of the stainless steel metal due to its ability to form chromium carbides ($Cr_{23}C_6$).[6] The carbides deplete the chromium content of the metal and, in turn, facilitate corrosion making trace amounts of carbon in stainless steel essential.

When biomaterials are implanted into the human body, another problem that may arise includes non-specific protein and cell attachment[3] and infection.[7] This research focuses on eliminating the short and long term effects of non-specific protein and cell attachment to biomaterials such as stents that are placed within the body's vascular system. These problems include restenosis (reclosure of the artery) and thrombosis (blood clot formation).[8, 9] Restenosis stems from the inability of the body's immune system to recognize the implant because they are not analogous to any tissues found within the body.[1, 4] An implant is considered to be a foreign object to the body's immune system and a wound healing response is triggered upon implantation.[4] Scar tissue cells such as lymphocytes are sent to the site of the implanted biomaterial in order to attack the unwanted object.[3] The cells attach to the surface which in turn forms fibrous tissue,

encasulating the implant, and the biomaterial cannot perform the intended task for which it was implanted.[3] Not only is revision surgery necessary, but the patient feels pain, and inflammation at the site is typical. Thrombosis occurs when blood cells such as platelets adhere to the implant and cause coagulation of the blood on the material.

One way to eliminate non-specific cell attachment is to modify the surface of the metals, in turn altering the physical and chemical properties of surface/air interface of the materials. There are a variety of methods to modify surfaces including Langmuir-Blodgett films, multilayer films, and self-assembled monolayers (SAMs).[1, 2, 4, 6] SAMs are advantageous over other methods because they are a single-layered protective coating which is strongly bound to the metal/metal oxide surface.[2, 4, 6] Self-assembled monolayers are also synthetically flexible and form spontaneously on a surface when under mild solution deposition conditions[6] (Figure 3) making them attractive for industrial use.

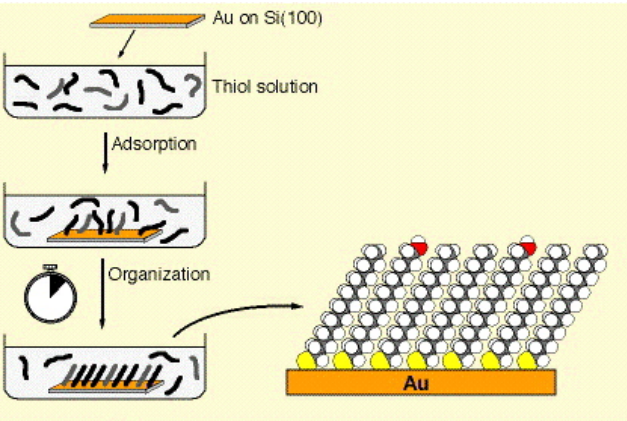


Figure 3: Spontaneous formation of organized monolayers from a solution onto a gold surface.

SAMs consist of a head group, C_n alkyl chain, and a tail group. The head group of the SAM interacts with the metal substrate, the alkyl chain determines the molecular ordering of the monolayer on the surface (Figure 4) and the tail moiety interacts with the surrounding environment.[1, 2, 6] SAMs potentially provide the ability to form surfaces that mitigate the non-specific attachment of cells by allowing for the control of the moiety present at the surface/air interface which would in turn allow for control of the interaction between the surface and the biological environment.

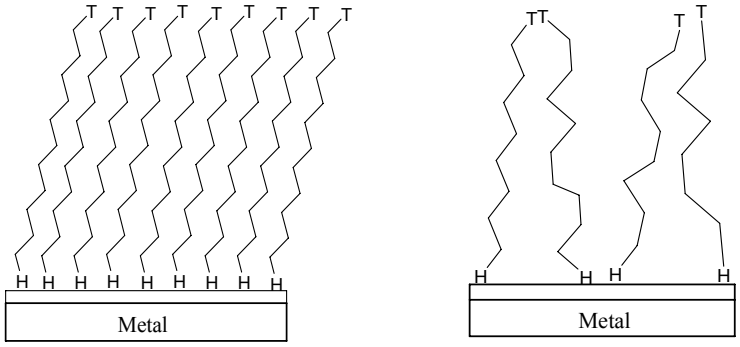


Figure 4: An illustration of an ordered monolayer (left) and a disordered monolayer (right) on a metal oxide surface. H is representative of the head group attached to the oxide surface. T is representative of the tail group which functions with the surroundings. Order occurs when the molecules are aligned on the surface in an all-trans configuration while disordered monolayers include gauche interactions in the alkyl chain.

SAMs can be altered to vary the interaction of the molecule with the surface, order of the alkyl chain, and the interfacial properties of a biomaterial. The head group of a SAM can be changed from a thiol to a carboxylic acid, phosphonic acid, etc. in order to enhance the surface/molecule interactions on a variety of substrates.[2] The length of the

alkyl chain can be altered to control the alkyl chain order on the surface, allowing for control of the moiety present at the interface. Research shows that the most ordered monolayers contain alkyl chains of 8-18 carbon.[10-13] Also, the tail groups can be changed from hydrophilic to hydrophobic allowing for control of the wettability of the surface which may alter the interaction of the substrate with the biological environment.[14, 15]

Most of the studies involving SAMs have been focused on using alkanethiols on gold surfaces. Gold is an ideal substrate for the formation of SAMs because of its smooth surface and strong sulfur-gold interactions allowing the formation of a robust, ordered monolayer on the surface.[11, 13, 14, 16, 17]

The mitigation of non-specific cell and protein attachment on gold surfaces has been investigated using a variety of alkanethiol monolayers.[18-26] Prior to using SAMs to alter the interfacial properties of gold, polyethylene glycol molecules were grafted onto the gold surface to determine their ability to mitigate non-specific protein and cell attachment. The studies have rendered such surfaces to be inert,[27-32] however grafting the molecules onto the surface does not allow for molecular control over the surface-environment interactions; therefore, SAMs of alkanethiols presenting oligomer form of the polymer at the interface were investigated to determine their ability to mitigate non-specific protein and cell attachment.[27, 29, 30] By using SAMs, surface coverage and interfacial properties of the material could be controlled strategically. The majority of studies confirm that alkanethiols terminated with oligo(ethylene glycol) moieties such as triethylene glycol (Figure 5) provide surfaces that are inert to non-specific protein and cell adhesion.[14, 18, 19, 22, 25, 26, 33-37] Other tail groups which provide a bio-inert gold surface include the mannitol[23] and galactose[38] moieties (Figure 6), and phospholipid-terminated

SAMs,[39] however oligo(ethylene glycol)-terminated alkanethiols continue to be the standard for protein and cell mitigation.[40]

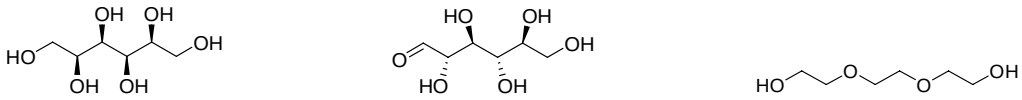


Figure 5: Structures of the three functional tail groups of inert gold surfaces: mannitol, galactose, and tri(ethylene glycol).

While gold is an ideal substrate to study the process of SAM formation on a metal surface, it is inconvenient to use as a practical biomaterial. Gold is not durable over long periods of time, and is also expensive when compared to other metals like stainless steel. As mentioned previously, SS316L is an actual material used to make arterial stents; therefore, it is desirable to study the modification of such surfaces with SAMs to determine an interface that is able to mitigate cell attachment on practical materials. While thiols form a strong interaction with gold surfaces, there are no interactions between thiols and metal oxides; therefore, the chemistry of forming alkanethiol SAMs on gold is not transferable metals that contain the native oxide layer on the surface.[41] The head groups of the molecules need to be altered from a thiol to an acid in order to chemisorb onto the native oxide layer of the non-ideal metal because the reactive hydroxyl and μ-oxo groups on the surface form bonds with acidic head groups, in turn, leading to SAM formation.[11, 42-44] The binding of carboxylic[11, 41, 42, 45] and phosphonic[43, 45-48] acids on metal oxides have been investigated and show the ability to chemisorb onto the native oxide surfaces of metals such as aluminum and stainless steel making them desirable head group functionalities for this study.

1.2 Thesis Goals

Objectives: Oligo(ethylene glycol)-terminated molecules with carboxylic and phosphonic acid head groups were synthesized and used to form self-assembled monolayers on the native oxide of stainless steel 316L along with methyl-terminated carboxylic and phosphonic acids. Modified surfaces were incubated with 3T3 fibroblast cells for twenty four hours and analyzed to determine which surface provided the greatest reduction in non-specific cell attachment.

Hypothesis: Self-assembled monolayers terminated with oligo(ethylene glycol) moieties will provide the most inert surfaces when compared to control and methyl-terminated monolayers.

Rationale: Oligo(ethylene glycol)-terminated SAMs have become the standard for protein and cell resistance on gold and silicon surfaces due to their ability to mitigate the non-specific attachment of proteins and cells. While the head group of the monolayer must be altered from a thiol to an acid in order to modify the SS316L surface, the group present at the surface/air interface will not be changed; therefore, similar results are expected on SS316L substrates.

Chapter 2: Synthesis of Oligo(ethylene glycol)-terminated Carboxylic Acids and Monolayer Formation on Stainless Steel 316L

2.1 Introduction

SS316L is a standard metal oxide used for biomaterial implants, especially stents. One of the problems associated with stents is restenosis, reclosure of the artery, due to non-specific cell adhesion on the implantation. It is proposed that in order to mitigate such a problem, SAMs of long chain carboxylic and phosphonic acids with varying tail groups can be attached to the surface of the SS316L. The tail group determines the hydrophilicity of the surface, which allows for control of the interfacial properties of the material potentially leading to mitigation of unwanted cells from the surface.

It has been shown in the literature on model surfaces, such as gold, that both grafted polyethylene glycol and SAMs terminated with (oligo)ethylene glycol form the most inert surfaces.[14, 18, 33, 35, 49] However, thiols do not react with the SS316L to form SAMs and polyethylene glycol cannot be directly grafted to the surface. It has been found that carboxylic[11, 42, 45] and phosphonic[43, 46-48] acids covalently attach to the surface of the SS316L in a closely-packed and ordered fashion; therefore, carboxylic and phosphonic acids terminated with tri- and pentaethylene glycol were synthesized in order to functionalize the SS136L surface. The syntheses were characterized by proton nuclear magnetic resonance spectroscopy (^{1}H NMR), fourier transform infrared spectroscopy (FTIR), and electrospray mass spectrometry (ESI MS).

Once the acids were synthesized, they were used to form SAMs on the SS316L surface along with methyl-terminated long chain carboxylic and phosphonic acids using a variety of solution deposition conditions. The modified surfaces were rinsed and sonicated in THF to remove and multilayers and/or weakly adsorbed materials, and characterized using diffuse reflectance fourier transform infrared spectroscopy (DRIFT),

contact angle analysis and matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

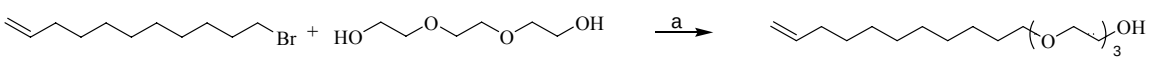
2.2 Materials

SS316L foils (0.5 mm, 99.99% pure, % wt : Fe 66%, Cr 19%, Ni 10 %, Mn 3% and Mo 2%) were obtained from Goodfellow Inc. THF, methanol, octadecylphosphonic acid (98%), octacosylcarboxylic acid (98%), triethyl phosphite (98%), hydrobromic acid (48%), 11-bromo-1-undecene (98%), triethylene glycol (99%), pentaethylene glycol (98%), 12-bromododecanoic acid (97%), chlorotrimethylsilane (97%), and sodium iodide (ACS reagent grade) were obtained from Aldrich Chemical Co. and were used without further purification. Acetonitrile (HPLC grade), hydrogen peroxide (30%), concentrated hydrochloric acid, sodium hydroxide, hexanes, chloroform and methylene chloride were obtained from Fisher Scientific and used without further purification. Anhydrous dichloromethane was obtained from Acros.

2.3 Synthesis

2.3.1. 11-tri(ethylene glycol)undecylphosphonic acid

a. Formation of 11-tri(ethylene glycol)-1-undecene

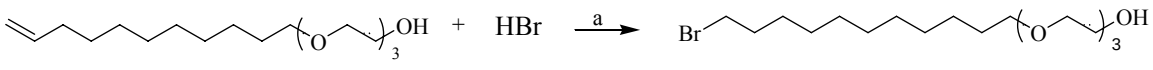


Scheme 1: Formation of 11-tri(ethylene glycol)undecene (65-78% yield). a= 50% aqueous NaOH, 60°C, N_2, 18 hours

A silicon oil bath was heated for fifteen minutes until the temperature reached 100°C. Five equivalents of triethylene glycol and one equivalent of 50% sodium hydroxide were added to a three-neck round bottom flask. The flask was placed into the heated oil bath, and the mixture reacts under nitrogen and heat for 30 minutes. One equivalent of 11-bromo-1-undecene was added to the flask, and the mixture was continuously heated and stirred under N_2 for 12-24 hours. The reaction was removed from the heat, allowed to cool to room temperature and extracted with hexanes and deionized water. The organic layer was dried with magnesium sulfate (Acros), vacuum filtered, and the solvent was evacuated leaving a pale yellow oily residue. Column chromatography was utilized for purification of the compound using a 2:1 ethyl acetate:hexanes mobile phase and a flash silica gel stationary phase. 11-bromoundecene was the first to emerge followed by the product then triethylene glycol. A pale yellow oily residue remained after evacuation of the solvent.

δ: 1.3ppm= 12H, triplet, methylene; 2.0ppm= 2H, quartet, methylene; 3.4ppm= 2H, triplet, methylene; 3.6ppm= 10H, triplet, methylene; 3.7ppm= 2H, triplet, CH_2; 4.9ppm= 2H, triplet, ethylene; 5.8ppm= 1H, triplet, ethylene

b. Formation of 11-tri(ethylene glycol)-1-bromoundecane



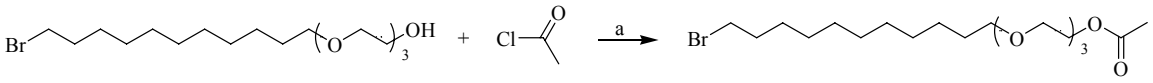
Scheme 2. Formation of 11-tri(ethylene glycol)-1-bromoundecane (82-88% yield).

a= H_2O_2, 55°C, UV light, 2 hours

A silicon oil bath was heated for fifteen minutes until the temperature reached 100°C. Four equivalents of 30% hydrogen peroxide was added to a round bottom flask and placed into the oil bath for five minutes under ultraviolet radiation. Ten equivalents of concentrated hydrobromic acid and one equivalent of (a) were added to the flask. The mixture was continuously heated and stirred for two hours to ensure completion, cooled to room temperature, then extracted with hexanes and water. The organic layer was dried with magnesium sulfate (Acros), vacuum filtered, and the solvent was evacuated leaving a clear oily residue in the flask.

δ: 1.3ppm= 12H, triplet, methylene; 1.5ppm= 2H, quintet, methylene; 1.8ppm= 2H, quintet, methylene; 3.3ppm= 2H, triplet, methylene; 3.4ppm= 2H, triplet, methylene; 3.5ppm= 2H, singlet, methylene; 3.6= 8H, triplet, methylene; 3.8ppm= 2H, triplet, methylene

c. Protection of the terminal hydroxyl group



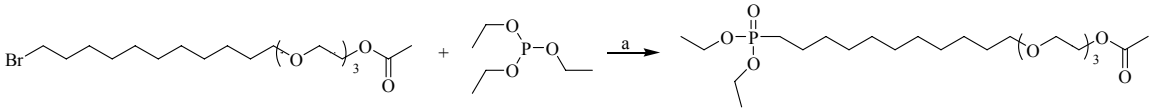
Scheme 3. Protection of the terminal hydroxyl group (85-90% yield). a= 0°C, N_2, 15 min

Product (b) was dissolved into 25mL of anhydrous dichloromethane. The solution was transferred to a round bottom flask, and equilibrated in an ice bath (0°C) under N_2 for fifteen minutes. Two equivalents of acetyl chloride were added dropwise over a fifteen minute interval while the reaction was continuously stirred at 0°C under N_2. The reaction is removed from the ice bath, warmed to room temperature, and the product

is extracted with methylene chloride and water. The organic layer was dried with sodium sulfate (Fisher), vacuum filtered, and the solvent was evacuated.

δ: 1.3ppm= 12H, triplet, methylene; 1.5ppm= 2H, quintet, methylene; 1.8ppm= 2H, quintet, methylene; 2.1ppm= 3H, singlet, methyl; 3.3ppm= 2H, triplet, methylene; 3.4ppm= 2H, methylene, triplet; 3.6ppm= 8H, triplet, methylene; 3.8ppm= 2H, triplet, methylene; 4.2ppm= 2H triplet, methylene

d. Addition of triethyl phosphite

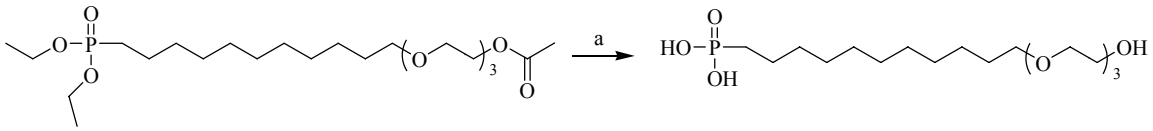


Scheme 4. Addition of triethyl phosphite (70-80% yield). a= 150°C, reflux, 15 hours

A silicon oil bath was heated until the temperature reached 150°C. Product (c) and six equivalents of triethyl phosphite were combined in a round bottom flask and set in the heated oil bath. The reaction refluxed at 150°C for fifteen hours then was removed from the heat and cooled to room temperature. Excess unreacted triethyl phosphite was removed by vacuum distillation leaving a yellow oily residue.

δ: 1.2ppm= 6H, triplet, methyl; 1.3ppm= 14H, multiplet, methylene; 1.5ppm= 2H, quintet, methylene; 1.8ppm= 2H, triplet, methylene; 2.0ppm= 3H, singlet, methyl; 3.4ppm= 2H, triplet, methylene; 3.6ppm= 8H, triplet, methylene; 3.8ppm= 2H, triplet, methylene; 4.0ppm= 4H, quartet, methylene; 4.2ppm= 2H, triplet, methylene

e. Deprotection to form 11-tri(ethylene glycol)undecylphosphonic acid



Scheme 5: Deprotection to form 11-tri(ethylene glycol)undecylphosphonic acid (77-89% yield, cumulative yield of 35-45%). a= ACN, sodium iodide, chlorotrimethylsilane, N_2, 16 hours

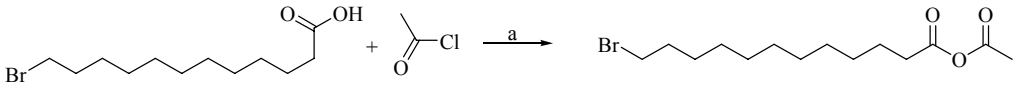
Product (d) was added to a round bottom flask containing one equivalent of dry acetonitrile, and the mixture was purged under nitrogen for fifteen minutes. Sodium iodide (2.2 equivalents) was added to the flask and allowed to dissolve completely for approximately five minutes under nitrogen. Then the dropwise addition of two equivalents of chlorotrimethylsilane occured over a fifteen minute interval and yielded a red/brown suspension immediately. The mixture reacted for two additional hours at room temperature under nitrogen. The acetonitrile was removed by vacuum, a 50/50 acetone/water mixture was added to the round bottom flask, and the mixture was stirred for one hour. The acetone was removed by vacuum, the water concentration was doubled, and the product was extracted with chloroform. The organic layer was back-extracted with water, dried with sodium sulfate, vacuum filtered, and evacuated to yield 11-tri(ethylene glycol)undecylphosphonic acid, which was an oily brown residue.

δ: 1.3ppm= 16H, multiplet, methylene; 1.5ppm= 2H, triplet, methylene; 1.7ppm= 2H, triplet, methylene; 3.4ppm= 2H, triplet, methylene; 3.5ppm= 8H, singlet, methylene;

3.6ppm= 2H, triplet, methylene; 3.7ppm= 2H, triplet, methylene; 5.0ppm= 3H, singlet, alcohol

2.3.2 12-tri(ethylene glycol)dodecylcarboxylic acid

a. Formation of 12-bromododecanhydride

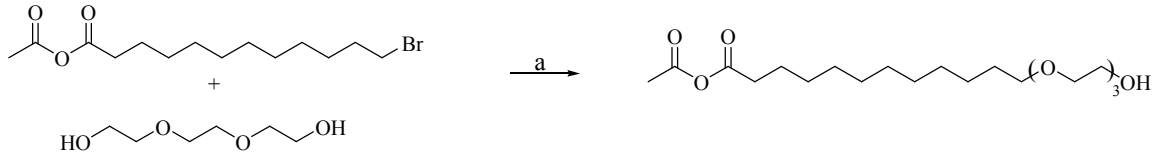


Scheme 6: Formation of 1-bromododecanhydride. a= 0˚C, N_2

The synthetic scheme for the formation of 12-bromododecanhydride is shown in Scheme 6. 12-bromododecanoic acid was dissolved in twenty five milliliters of methylene chloride, added to a round-bottom flask, and equilibrated in an ice bath (0°C) under nitrogen for fifteen minutes. Two equivalents of acetyl chloride were added dropwise over a fifteen minute interval while the flask remained in the ice bath under nitrogen. After the addition of acetyl chloride, the product reacted for an additional hour at 0°C under N_2. The reaction was warmed to room temperature and extracted with methylene chloride and water. The organic layer was dried with sodium sulfate, vacuum filtered, and the solvent was evacuated leaving a white powder.

δ: 1.2ppm= 14H, triplet, methylene; 1.6ppm= 2H, quintet, methylene; 1.8ppm= 2H, quintet, methylene; 2.1ppm= 3H, singlet, methyl; 2.3ppm= 2H, triplet, methylene; 3.3ppm= 2H, triplet, methylene

b. Addition of triethylene glycol to 12-bromododecanhydride

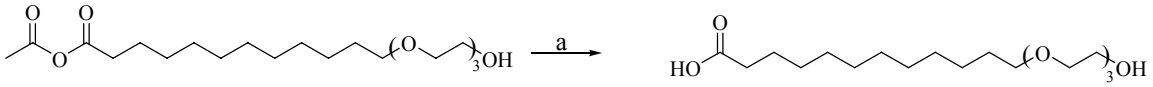


Scheme 7: Addition of triethylene glycol to 12-bromododecanhydride. a= 100˚C, N_2, 12-24hrs.

Five equivalents of triethylene glycol and one equivalent of 50% sodium hydroxide were reacted for thirty minutes in a heated oil bath (T=100°C) under nitrogen. Product (a) was dissolved into twenty five milliliters of methylene chloride and added to the mixture which was continuously heated and stirred for 12-24 hours under nitrogen. The reaction was cooled to room temperature, and the product was extracted with hexanes and water. The organic layer was dried with magnesium sulfate, vacuum filtered, and the solvent was evacuated to yield a white powder.

δ: 1.3ppm= 14H, triplet, methylene; 1.5ppm= 2H, quintet, methylene; 1.6ppm= 2H, quintet, methylene; 3.4ppm= 2H, triplet, methylene; 2.2ppm= 3H, singlet, methyl; 2.3ppm= 2H, triplet, methylene; 3.5ppm= 8H, multiplet, methylene; 3.6ppm= 2H, triplet, methylene, 3.8ppm= 2H, triplet, methylene

c. Deprotection to form 12-tri(ethylene glycol)dodecylcarboxylic acid



Scheme 8. Deprotection to form 12-tri(ethylene glycol)dodecylcarboxylic acid. a= 6M HCl, 100˚C

A silicon oil bath was heated until the temperature reached 100°C. Product (b) was added to a round bottom flask and placed into the heated oil bath. Concentrated hydrochloric acid was diluted to a 6M working concentration using deionized water then poured into the round bottom flask containing product 4. The reaction was refluxed for seventy two hours, warmed to room temperature, and extracted with additional water and methylene chloride. The organic layer was dried with sodium sulfate, vacuum filtered, and solvent was evacuated to yield crude 12-tri(ethylene glycol)dodecylcarboxylic acid, which was a clear oily residue. Column chromatography was used to purify the compound using a 2:1 ethyl acetate:hexanes mobile phase and a silica gel stationary phase. The pure product was third to emerge from the column and a sticky white residue remained after evacuation of the solvent.

δ: 1.3ppm= 14H, triplet, methylene; 1.5ppm= 2H, quintet, methylene; 1.6ppm= 2H, quintet, methylene; 2.3ppm= 2H, triplet, methylene; 3.4ppm= 2H, triplet, methylene; 3.5ppm= 8H, singlet, methylene; 3.6ppm= 2H, triplet, methylene; 3.7ppm= 2H, triplet, methylene

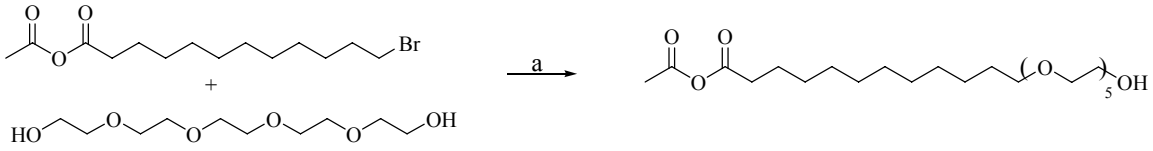
2.3.3 12-penta(ethylene glycol)dodecylcarboxylic acid

a. Formation of 12-bromododecanhydride

12-bromododecanoic acid was dissolved in twenty five milliliters of methylene chloride, added to a round-bottom flask, and equilibrated in an ice bath (0°C) under nitrogen for fifteen minutes. Two equivalents of acetyl chloride were added dropwise over a fifteen minute interval while the flask remained in the ice bath under nitrogen. After the addition of acetyl chloride, the product reacted for an additional hour under N_2 at T=0°C. The reaction was warmed to room temperature and extracted with methylene chloride and water. The organic layer was dried with sodium sulfate, vacuum filtered, and the solvent was evacuated yielding a white powder.

δ: 1.2ppm= 14H, triplet, methylene; 1.6ppm= 2H, quintet, methylene; 1.8ppm= 2H, quintet, methylene; 2.1ppm= 3H, singlet, methyl; 2.3ppm= 2H, triplet, methylene; 3.3ppm= 2H, triplet, methylene

b. Addition of pentaethylene glycol to 12-bromododecanhydride



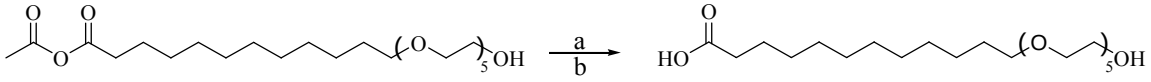
Scheme 9: Addition of pentaethylene glycol to 12-bromododecanhydride. a= 65˚C, N_2, 12-24hrs.

Five equivalents of pentaethylene glycol and one equivalent of 50% sodium hydroxide were reacted for thirty minutes in a heated oil bath (T=55°C) under nitrogen.

Product (a) was dissolved into twenty five milliliters of methylene chloride and added to the mixture. The reaction was continuously heated and stirred for 12-24 hours under nitrogen. The reaction was then cooled to room temperature and extracted with hexanes and water. The organic layer was dried with magnesium sulfate, vacuum filtered, and the solvent was evacuated yielding a white powder.

δ: 1.2ppm= 14H, quintet, methylene; 1.6ppm= 4H, multiplet, methylene; 2.0ppm= 3H, singlet, methyl; 2.3ppm= 2H, triplet, methylene; 3.4ppm= 2H, triplet, methylene; 3.6ppm= 16H, singlet, methylene; 3.7ppm= 2H, triplet, methylene; 4.2= 2H, triplet, methylene

c. Deprotection to form 12-penta(ethylene glycol)dodecanecarboxylic acid



Scheme 10. Deprotection to form 12-penta(ethylene glycol)dodecylcarboxylic acid. a= 2M NaOH, 100˚C, N_2; b= 6M HCl

Product (b) was deprotected by a base deprotection method. Thirteen equivalents of 2M NaOH were heated until T=100˚C and purged under nitrogen for one hour. Product (2) was then dissolved into methylene chloride and added to the purged NaOH solution. The mixture was heated and stirred continuously under nitrogen for three hours. The reaction was allowed to cool to room temperature. Ten milliliters of 6M HCl was added to precipitate the white powder product which was then vacuum filtered and dried at room temperature overnight before further analysis.

δ: 1.3ppm= 14H, multiplet, methylene; 1.5ppm= 4H, multiplet, methylene; 2.3ppm= 2H, triplet, methylene; 3.4ppm= 2H,, triplet, methylene; 3.5ppm= 16H, singlet, methylene; 3.6ppm= 2H, triplet, methylene; 3.8ppm= 2H, triplet, methylene

2.4 Synthetic Analysis Methods

2.4.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

All ^{1}H NMR spectra were acquired using a 300 MHz Bruker spectrometer in one dimension. NMR spectroscopy was used in order to determine structural information on each synthetic step of both 11-tri(ethylene glycol)undecylphosphonic acid and 12-tri(ethylene glycol)dodecanoic acid. Individual products were dissolved at a concentration of approximately 600μM into deuterated chloroform (Aldrich, 99.8 atom% D) which was used as a reference solvent.

2.4.2 Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR Spectroscopy was used in conjunction with NMR spectroscopy and Mass Spectrometry (MS) to verify the final syntheses. FTIR analysis was performed using a Thermo Nicolet-NEXUS 470 FT-IR spectrometer in transmission mode. The final product of both the 11-tri(ethylene glycol)undecylphosphonic acid, 12-tri(ethylene glycol)dodecanecarboxylic acid, and 12-penta(ethylene glycol)dodecanecarboxylic acid syntheses were analyzed using potassium chloride salt plates. A drop of each product was sandwiched between two KCl salt plates in order to spread the products into a thin layer. A background spectrum was obtained in order to subtract any peaks due to the salt

plates and the environment, and the spectra obtained were plotted as %Transmittance vs. frequency (cm^{-1}).

2.4.3 Electrospray Mass Spectrometry (ESI MS)

ESI MS was used in order to determine the purity and mass to charge ratio (m/z) of the 11-tri(ethylene glycol)undecylphosphonic acid, 12-tri(ethylene glycol)dodecane carboxylic acid acid and 12-penta(ethylene glycol)dodecanecarboxylic acid final products. Spectra were recorded on a Micromass ZMD spectrometer in positive electrospray mode. All acids were dissolved into methanol at a concentration of 0.1mM. Samples were injected into the MS at a rate of 150μL/min. Spectra were obtained over a 5 minute interval.

2.5 Monolayer Formation

2.5.1 Substrate Preparation

SS316L foil was sanded using 150, 400, and 600 grit sandpaper and cut into 1x1 cm samples. Substrates were cleaned by rinsing with acetone and methanol, sonicating in methanol for 30 minutes, heating in methanol for 10 minutes, and drying in a 100°C oven overnight.

2.5.2 Monolayer Deposition

a) Aerosol Deposition

Self-assembled monolayers of octadecylphosphonic acid (CH_3-P, 1mM) and 12-tri(ethylene glycol)dodecylcarboxylic acid (EG3-C, 2mM) were formed using an aerosol

deposition method. This method consisted of a TLC sprayer filled with a solution of the respective acid and connected to a nitrogen flow. The clean, room temperature substrates were sprayed one time for CH3-P and five times for EG3-C. The substrates were either stored in a 100ºC oven (CH_3-P) or under vacuum (EG3-C) for approximately one half hour between sprays. The samples were then stored overnight before further analysis. All samples were rinsed and sonicated in THF to remove any weakly adsorbed material.

b) Dip Deposition

SAMs of octacosylcarboxylic acid (CH_3-C) and 11-tri(ethylene glycol)undecylphosphonic acid (EG3-P) were prepared by placing clean substrates in an ice bath for 1 hr, dipping in a warm (55°C) 2mM THF solution of the respective acid for 2 hours and stored in an oven at 100°C overnight before further analysis. All samples were rinsed and sonicated in THF to remove any weakly adsorbed material.

2.6 Monolayer Characterization

2.6.1 Diffuse-Reflectance Fourier Transform Infrared Spectroscopy (DRIFT)

Diffuse-Reflectance Fourier Transform Infrared Spectroscopy (DRIFT) spectroscopy was used to determine molecular adsorption and alkyl chain ordering on the surface using a Thermo Nicolet-NEXUS 470 FT-IR spectrometer. Spectra were taken using a diffuse reflectance attachment for 1024 scans. Nitrogen was used to eliminate background noise due to atmospheric molecules. The molecular order on the surface was determined by the numerical value of the CH_2 anti-symmetric and symmetric peaks of the molecule. The binding region of the spectra (1800-500cm^{-1}) was used to verify whether the acid was covalently attached to the surface.

2.6.2 Contact Angle

Contact Angle goniometry was performed on a VCA Optima Goniometer located at the University of Pittsburgh (Pittsburgh, PA). The wettability of the modified and unmodified SS316L substrates was determined by the method of Bain.[50] A one microliter droplet of deionized (Millipore) water brought into contact with the surfaces and the angle measurements on three different places of three different samples were taken and the averages and standard deviations were calculated. A higher contact angle value was indicative of a hydrophobic surface while a lower contact angle value was indicative of a hydrophilic surface.

2.6.3 Atmospheric Pressure Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (AP MALDI-TOF MS)

Atmospheric pressure matrix-assisted laser desporption/ionization time-of-flight mass spectrometry was performed on an AP-MALDI TOF mass spectrometer from Agilent Technologies in order to characterize monolayer formation on SS316L. The matrix, α-cyano-4-hydrocinamic acid (CHCA), was purchased from Sigma-Aldrich (>99.0% purity) and used without further purification. CHCA was diluted to a concentration of 10mg/mL, added to the surface of the modified substrates, and allowed to air dry. Double-sided tape was used to hold the substrates in place on the sample plate. Ions were detected in positive mode with a capillary voltage of 3500V, fragmentor voltage of 260V, skimmer voltage of 40V and drying temperature of 325º C over a 1-5 minute interval. Spectra for three different spots on each sample were acquired.

2.7 Results and Discussion

2.7.1 Synthesis

The use of a variety of characterization methods allows for full characterization of product formation; therefore ^{1}H NMR, FTIR, and ESI MS were used to analyze product formation. Individual steps of the syntheses were analyzed using ^{1}H NMR spectroscopy to determine specific product formation. The final products were analyzed using ^{1}H NMR along with FTIR and ESI MS to provide a complete investigation of the overall syntheses.

a. 11-tri(ethylene glycol)undecylphosphonic acid

The results from ^{1}H NMR are given in the materials and methods section and indicate the successful synthesis of 11-tri(ethylene glycol)undecylphosphonic acid. The results were verified using FTIR and also ESI MS. The FTIR spectrum seen in Figure 7 indicated the presence of the final product with the presence of an –OH functional group at 3390 cm^{-1}, CH_2 antisymmetric and symmetric stretches at 2924 and 2853 cm^{-1} respectively, a P=O stretch at 1460 cm^{-1}, a P-O stretch at 1104 cm^{-1}, and a P-O-H stretch at 984 cm^{-1}. The molecular weight of the final product is 384.54 amu, and the ESI MS spectrum also seen in Figure 6 further indicated formation of product due to the presence of the peak at 385.79 amu, which denotes the presence of the protonated product. The combination of analyses confirm the synthesis of 11-tri(ethylene glycol)undecylphosphonic acid.

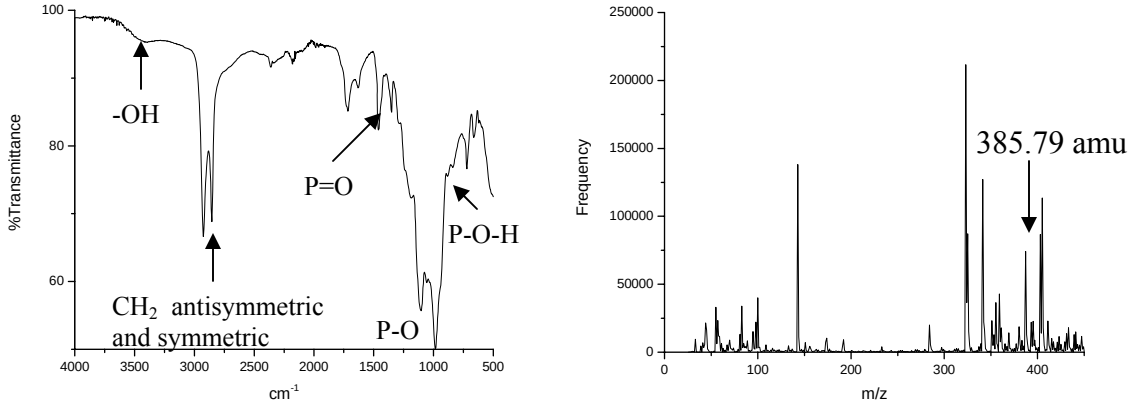


Figure 6: FTIR and ESI MS results for the synthesis of 11-tri(ethylene glycol)undecyl phosphonic acid. Stretches due to the presence of –OH, sp^3 C-H anti- and symmetric stretches, P=O, P-O and P-O-H stretches in the FTIR spectrum are indicative of product formation, and the peak on the ESI MS spectrum at 385.79 amu denotes the protonated product.

b. 12-tri(ethylene glycol)dodecylcarboxylic acid

The ^{1}H NMR results for 12-tri(ethylene glycol)dodecylcarboxylic acid are given in the material and methods section of this chapter and suggest the presence of the acid. The resulting product was further analyzed with FTIR and ESI MS analysis. The FTIR spectrum in Figure 7 shows the presence of –OH at 3364 cm^{-1}, sp^3 antisymmetric and symmetric stretches at 2916 and 2848 cm^{-1} respectively, a C=O stretch at 1724 cm^{-1}, and a C-O stretch at 1245 cm^{-1} which are all prominent peaks in the acid. Further indication of product formation can be seen in the ESI MS spectrum also shown in Figure 7 with the presence of the peak at 371.14 amu. The molecular weight of the final product is 348.51amu, and this peak denotes the sodiated product.

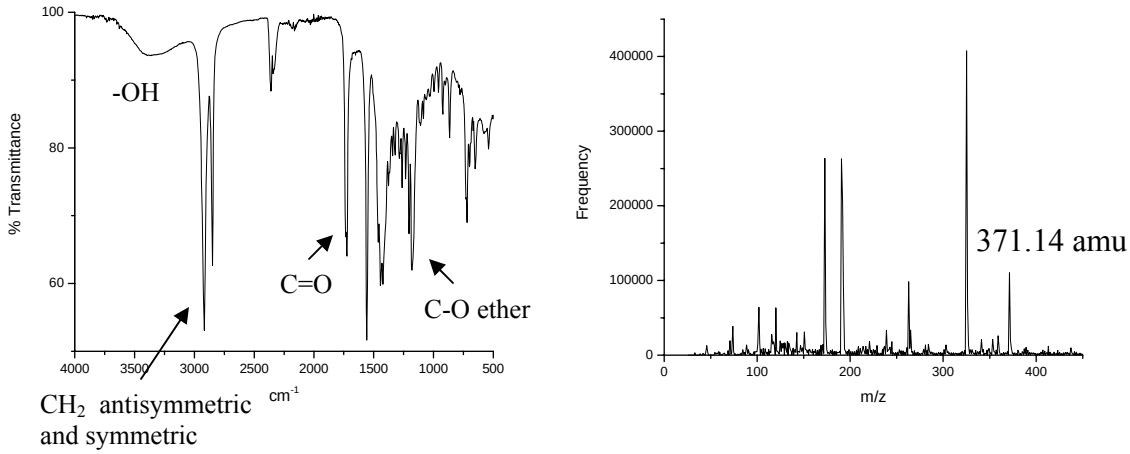


Figure 7: FTIR and ESI MS results for the synthesis of 12-tri(ethylene glycol)dodecyl carboxylic acid. Stretches due to the presence of –OH, sp^3 C-H anti- and symmetric stretches, C=O and C-O stretches in the FTIR spectrum are indicative of product formation, and the peak on the ESI MS spectrum at 371.14 amu denotes the sodiated product.

c. 12-penta(ethylene glycol)dodecylcarboxylic acid

The ^1H NMR results for 12-penta(ethylene glycol)dodecylcarboxylic acid are given in the material and methods section of this chapter and suggest the presence of the acid. The resulting product was further analyzed with FTIR and ESI MS analysis. The FTIR spectrum in Figure 8 shows the presence of –OH at 3364 cm^{-1}, sp^3 antisymmetric and symmetric stretches at 2916 and 2848 cm^{-1} respectively, a C=O stretch at 1724 cm^{-1}, and a C-O stretch at 1244 cm^{-1} which are all prominent peaks in the acid. Further indication of product formation can be seen in the ESI MS spectrum also shown in Figure 8 with the presence of the peak at 371.14 amu. The molecular weight of the final product is 348.51amu, and this peak denotes the sodiated product.

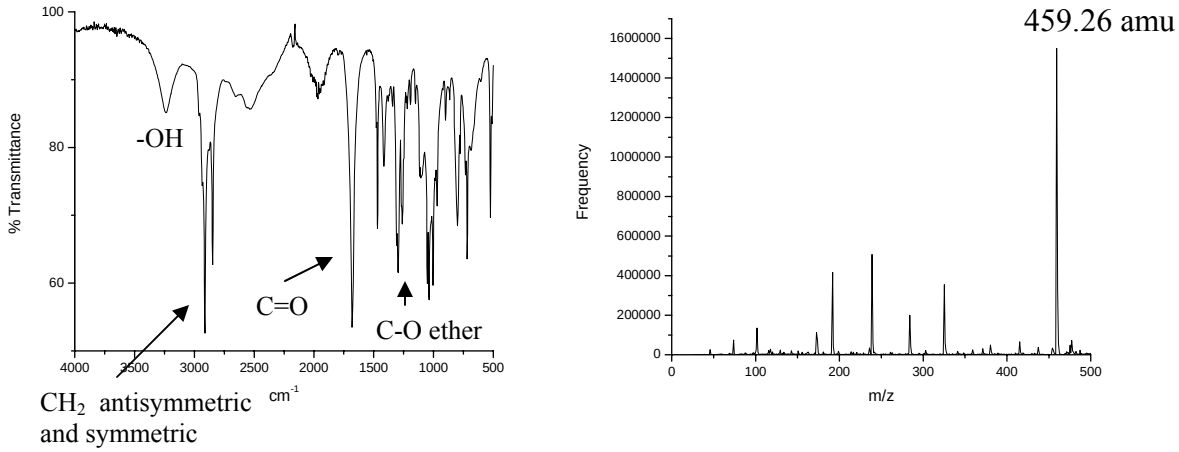


Figure 8: FTIR and ESI MS results for the synthesis of 12-penta(ethylene glycol)dodecyl carboxylic acid. Stretches due to the presence of –OH, sp^3 C-H anti- and symmetric stretches, C=O and C-O stretches in the FTIR spectrum are indicative of product formation, and the peak on the ESI MS spectrum at 459.26 amu denotes the sodiated product.

2.7.2 Monolayer Formation

Self-assembled monolayers (SAMs) were formed on the native oxide of SS316L substrates with both carboxylic and phosphonic acids utilizing aerosol and dip solution deposition methods. Substrates were then rinsed in THF for fifteen minutes and sonicated in THF for five minutes to remove loosely adhered material and determine stability of the monolayer. The resulting substrates were analyzed using DRIFT to determine molecular order and binding, contact angle goniometry to determine the wettability of the surface/air interface, and AP MALDI-TOF MS to determine mono- or multilayer formation on the surface.[51]

DRIFT Spectroscopy

DRIFT spectroscopy is a useful qualitative technique for the determination of molecular ordering and binding on the surface. Molecular ordering was determined by analyzing the CH_2 symmetric and anti-symmetric stretches. A molecule with an anti-symmetric stretch found at $\leq 2918 cm^{-1}$ and a symmetric stretch found at $\leq 2850 cm^{-1}$ was considered to be in an all-trans conformation on the surface and thus "ordered" while stretches below those values indicated monolayers that were disordered on the surface.[11, 35, 52] While it is important for the molecules to be ordered on the surface in order to present the proper tail moiety at the interface, it may not be necessary for the ethylene glycol molecules to be ordered due to the flexible nature of the moiety.

The CH_3-P, CH_3-C, EG3-P and EG3-C acids were used to form monolayers on the SS316L substrates. All of the modified substrates except for EG3-P had ordered monolayers on the surface with antisymmetric and symmetric stretches ranging from 2912-2915 cm^{-1} and 2847-2848 cm^{-1} respectively (Figure 9). EG3-P had antisymmetric and symmetric stretches at 2923 and 2852 cm^{-1} respectively and is considered to be a disordered monolayer on the surface. The EG3-P monolayer may appear to be disordered due to the flexibility of the glycol moieties present in the molecule. The C-O ether region of EG3-P is also shown in Figure 9 which verifies the presence of the triethylene glycol on the surface.

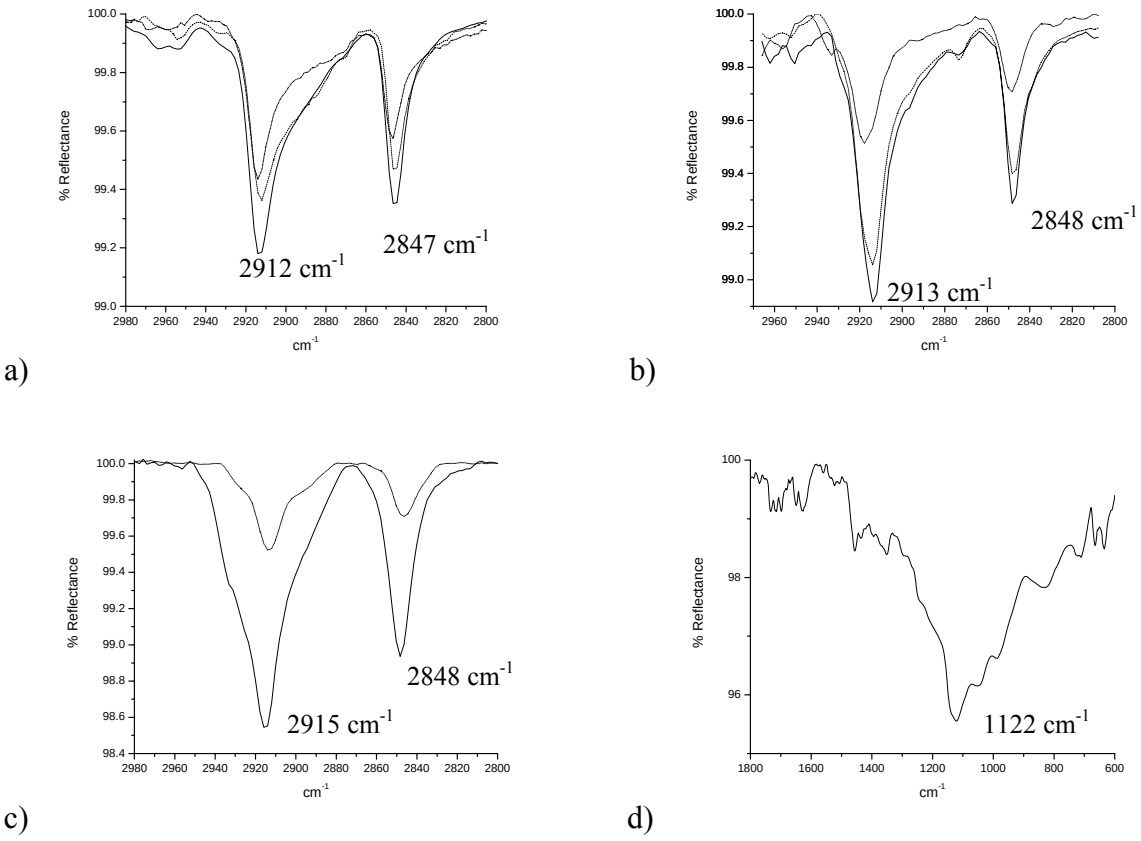


Figure 9: Formation of SAMs on the native oxide of SS316L using a) CH_3-C, b) CH_3-P, c) EG3-C, and d) Ether region of EG3-P. The CH_2 antisymmetric and symmetric stretches show an ordered monolayer after rinse and sonication. The presence of triethylene glycol in EG3-P is verified with the presence of the ether peak at 1122 cm^{-1}.

Binding of the organic acids to the SS316L surface was also investigated using DRIFT spectroscopy by analyzing the fingerprint region of the spectra. Binding of the phosphonic acids to the surface was determined by examining the P=O, P-O, and P-O-H stretches located at 1460, 1100, and 980 cm^{-1} respectively. A shift of the P-O-H stretch or disappearance of the P=O stretch would indicate monodentate binding on the surface

while the disappearance of the P-O-H stretch would indicate bidentate binding.[53] Tridentate binding would occur if both the P=O and P-O-H stretches were absent in the spectrum.[53] The phosphonic acids used demonstrate bidentate bonding to the SS316L surface, as suggested by the disappearance of the P-O-H stretch at 980 cm^{-1} seen in the spectrum for CH_3-P in Figure 10.

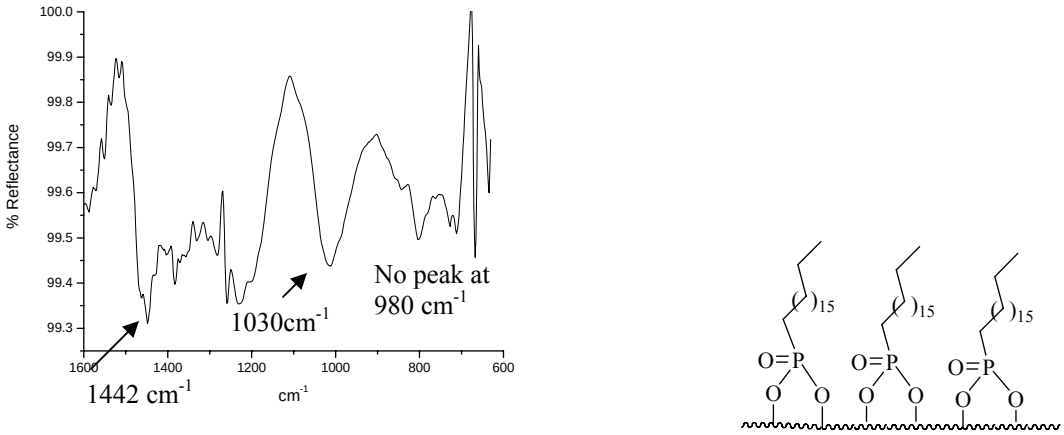


Figure 10: Binding region of CH_3-P monolayers on SS316L. Disappearance of the P-O-H stretch at 980 cm^{-1} strongly suggests bidentate binding to the surface.

The carboxylic acid spectra were also analyzed to determine the binding of the molecule to the surface by examining the C=O and C-O stretches at 1700 and 1240 cm^{-1} respectively. The appearance of the stretch at 1540 cm^{-1} in the carboxylic acid monolayer spectra suggests that the carboxylic acid monolayers bind in a bidentate fashion[54, 55] on the surface as seen in the spectrum for CH_3-C displayed in Figure 11 due to the disappearance of the C=O peak and the shift of the C-O peak. Additionally, the presence of a single peak indicates bidentate binding of the carboxylic acids to the surface.

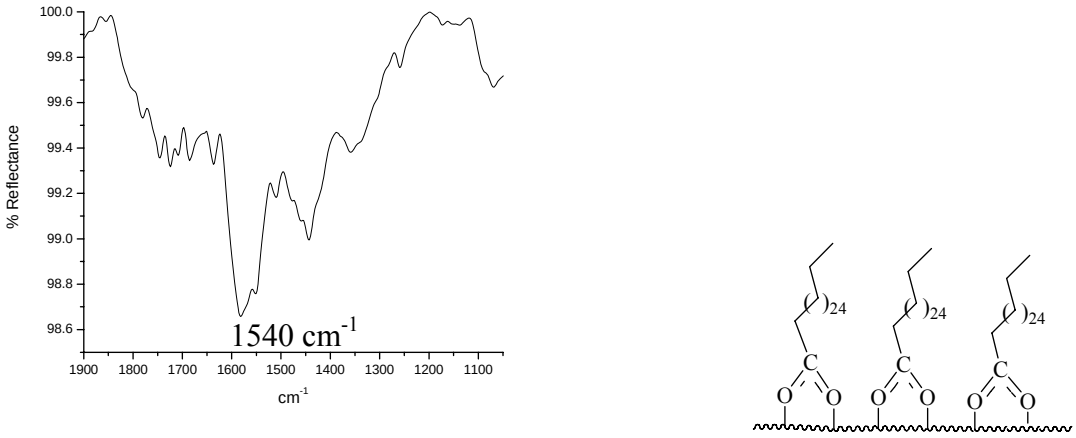


Figure11: Binding region of CH_3-C monolayers on SS316L. The presence of the peak at 1540 cm^{-1} strongly suggests bidentate bonding of the acid to the surface.

Contact Angle Goniometry

Contact angle goniometry was used to determine the wettability of the SS316L surface after modification with the different acids by examining the angle formed between the water droplet and the surface. A higher contact angle value was indicative of a hydrophobic surface while a lower contact angle value was indicative of a hydrophilic surface. Table 1 summarizes the molecular conformation and contact angle values for the control and modified surfaces.

Modification	FTIR Values (cm^{-1})	Contact Angle Values	Standard Deviation
SS316L control	n/a	53.6	3.0
CH_3-P	2913/2847	105.0	3.6
CH_3-C	2913/2847	109.9	4.2
EG3-C	2918/2849	76.5	5.9
EG3-P	2928/2853	78.3	3.8

Table 1: FTIR and contact angle results for the different acids used to modify SS316L. The methyl-terminated acids had higher values which suggest the formation of hydrophobic interfaces when water was brought into contact with the surface while the glycol-terminated molecules had water contact angles suggesting a mixture of hydrophobic and hydrophilic moieties at the interface.

Unmodified SS316L was used as a control and has a hydrophilic interface due to the hydroxyl and μ-oxo groups of the oxide layer coating the surface. The CH_3-P and CH_3-C acids, had contact angle values of 105.0˚ +/- 5.4˚ and 109.9˚ +/- 4.2˚, respectively, suggesting the presence of the methyl group at the interface. The EG3-C (ordered) and EG3-P (disordered) acids had contact angle values of 76.5˚ +/- 5.9˚ and 78.3˚ +/- 3.8˚ which suggest an interface that is a combination of hydrophilic and hydrophobic moieties. While one would expect the glycol values to be more hydrophilic due to the terminal alcohol, the flexible nature of the tail potentially exposes both hydrophobic and hydrophilic moieties at the interface, possibly causing an increase in the water contact angle on the surface.

Atmospheric Pressure Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (AP MALDI-TOF MS)

AP MALDI-TOF MS was used to distinguish between mono- and multilayer formation on the surface. Samples which had an intensity of ≤ 0.5% in the DRIFT spectrum were analyzed using AP MALDI-TOF MS to determine mono- or multilayer formation. Research shows that a MALDI spectrum that has only the monomer peak of the acid is considered to be a monolayer while the presence of a dimer peak indicates multilayer formation.[51]

AP MALDI-TOF spectra were acquired on three different spots on three different samples for each of the acids. The monomer and dimer regions were analyzed for the presence of peaks. The spectra reveal only the presence of monomer peaks for CH_3-P, EG3-P, and EG3-C (Figure 12) suggesting the formation of monolayers on the SS316L surface. Figure 12 also shows the dimer region for EG3-P, and only the monomer peak at 387.3 amu is present in the spectra. AP MALDI TOF MS data was unable to be obtained for CH_3-C due to peak interference from the α-cyano-4-hydroxycinnamic acid matrix used to obtain the spectra.

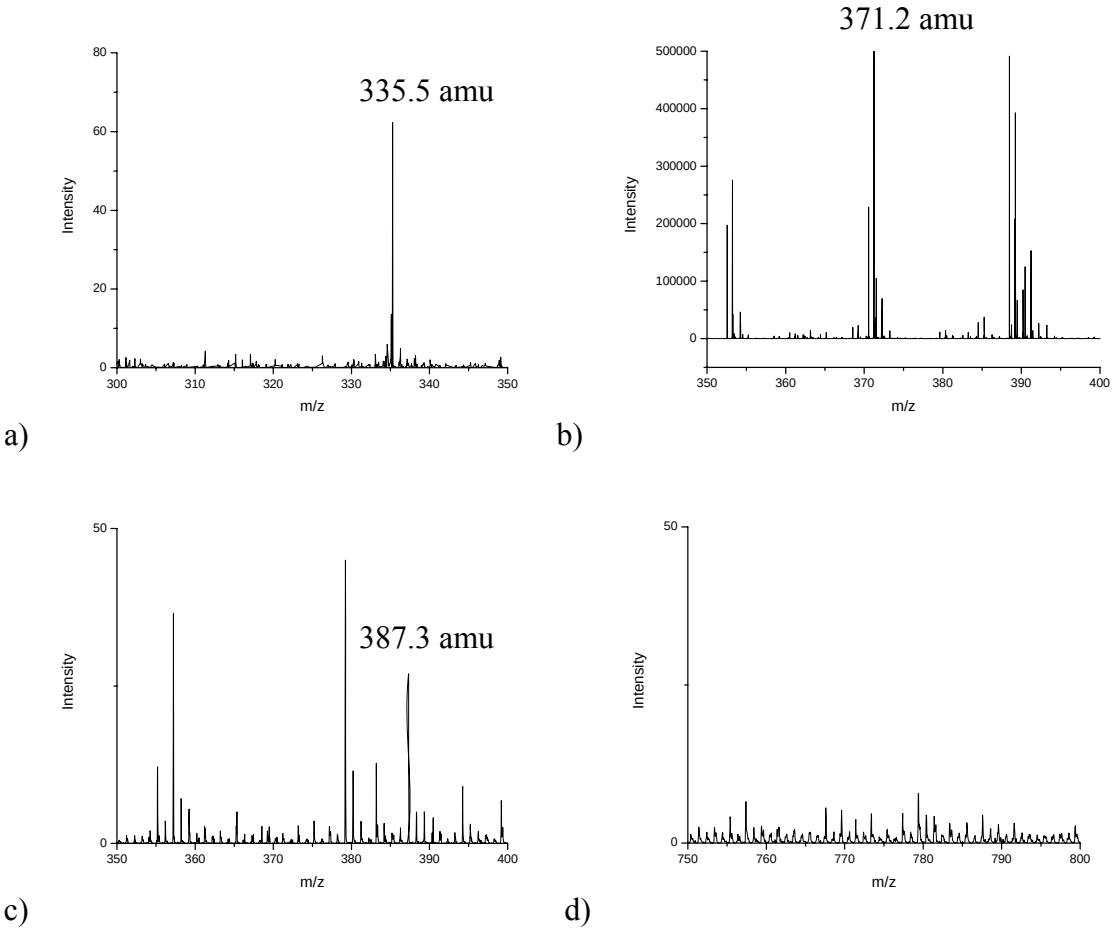


Figure 12: AP MALDI TOF images of the monomer region of a) CH_3-P b) EG3-C c) EG3-P and dimer region of d) EG3-P. The spectra reveal the presence of only the monomer peaks for each acid, indicating monolayer formation on the SS316L surface.

Chapter 3: Cell Attachment Studies and Conclusions

3.1 Introduction

When biomaterials such as stents are implanted in the body, problems may arise including thrombosis (blood clot formation) and restinosis (reclosure of the artery) due to non-specific adhesion of proteins and cells on the surface. [8, 9] One way to mitigate such problems is to modify the surface of the material with self-assembled monolayers (SAMs) presenting tail functionalities that will resist this non-specific adsorption. SAMs are desirable for use because they are synthetically flexible and spontaneously form on the surface under mild conditions. Synthetic flexibility allows for the variation of functional groups present at the surface/air interface to investigate surfaces that will resist non-specific adsorption. Most studies have shown that SAMs on gold and silicon which were terminated with oligo(ethylene glycol) moieties have provided the most inert surfaces while only a few show methyl-terminated SAMs mitigate non-specific adhesion most effectively.[56, 57]

In this study, the ability of methyl- and tri(ethylene glycol)-terminated carboxylic and phosphonic acid SAMs on SS316L to resist the non-specific attachment of 3T3 fibroblast cells was investigated. Octadecylphosphonic acid SAMs were chosen because previous experiments performed by Gawalt, et al,[58] conclude that these SAMs provided the most inert surfaces on SS316L when compared to other carboxylic and phosphonic acid SAMs of similar chain lengths terminated with hydroxyl, carboxylic acid, and amine functional groups. Research by Gawalt, et al, also showed that carboxylic and phosphonic acids on SS316L with short alkyl chains either do not form ordered monolayers (octylcarboxylic acid)[58] or are not ideal surfaces for the mitigation of fibroblast cells (octylphosphonic acid; octadecylcarboxylic acid);[58] therefore, the ability

of octacosylcarboxylic acid to mitigate fibroblast attachment was investigated. Longer alkyl chain lengths increase packing and order of the molecule on the surface which may be factors involved in the ability of an interface to mitigate fibroblast cells. 11-tri(ethylene glycol)undecylphosphonic acid and 12-tri(ethylene glycol)dodecyl carboxylic acid were chose to investigate whether triethylene glycol moieties provide inert surfaces on SS316L as they do on model substrates.

3.2 Materials

3T3 Swiss Albino mouse embryo fibroblast cells were obtained from ATCC (CCL-92). Dulbecco's modified eagle medium (DMEM), trypsin/EDTA, bovine calf serum, trypsin dissociation reagent 0.05% (1X), HyPure molecular biology grade water and penicillin/streptavidin solution (1%) were obtained from Fisher Scientific Co. The viability, cytotoxicity kit (L-3224) for mammalian cells was obtained from Invitrogen/Molecular Probes.

3.3 Cell Culture

The 3T3 fibroblast cells were cultured upon receipt from ATCC by thawing, suspending in complete medium consisting of DMEM, 10% calf serum and 1% penicillin/streptavidin, ultracentrifugation, then resuspending in the complete medium. The suspended cells were split into three T 25 flasks, maintained in culture in the complete medium, and incubated at 37˚C and 5% CO_2. They were passaged every three days and discarded after fifteen passages. Cells were removed from the flasks by trypsinization, washed once with DMEM, and resuspended in complete culture medium.

3.4 Cell Attachment Studies

3.4.1 Cell Seeding

The control and modified SS316L substrates were placed in a 24 well plate, sterilized with 70% ethanol for ten minutes, and rinsed three times using sterile distilled water. The sterilized substrates were left in the hood to dry completely before further use. The 3T3 fibroblast cells were then trypsinized (1X concentration) in order to detach the cells from the culture flask. The suspended 3T3 fibroblast cells were stained with trypan blue, counted using a haemocytometer and then seeded randomly at a density of 10,000 cells per well. After incubating the substrates with the cells in humidified air with 5% CO_2 at 37˚C for twenty four hours, the samples were rinsed three times with PBS buffer and analyzed by a viability/cytotoxicity assay.

3.4.2 Live/Dead Viability/Cytotoxicity Assay

A viability/cytotoxicity kit was used to provide a simultaneous determination of the presence of live and dead cells on the surface. The assay contained Calcein AM and ethidium homodimer dyes used to stain the live and dead cells respectively. The live cells were identified by the enzymatic conversion of the non-fluorescent cell permeate Calcein AM to intensely fluorescent calcein. Calcein is retained in live cells and produces a green fluorescence. The ethidium homodimer is excluded by the intact plasma membrane of the live cells but enters cells with damaged membranes (dead cells) where it binds to nucleic acid and produces a bright red fluorescence. Therefore, live cells fluoresced green and dead cells fluoresced red when viewed under a fluorescent microscope.

After incubating the substrates for twenty four hours in media containing cells, they were washed with PBS to remove any unattached cells, a half of a milliliter of the live/dead stain was added to each well and the well plate was covered with aluminum foil and incubated at 37˚C and 5% CO_2 for thirty minutes. Three samples of each type of modification were used for every set and three such sets were completed. Each sample was mounted on a microscope slide and viewed under a fluorescent Nikon Eclipse microscope at a 10X magnification. Cells were counted on five different spots on each sample, and the average value was used for statistical purposes. Viability calculations were performed on each sample by dividing the number of live cells by the total number of cells present.

3.4.3 Statistical Analysis

One-way ANOVA (analysis of variance) was used to determine the statistical difference between the means of data sets for cell experiments with the modified substrates and multiple comparisons of the sample means was performed using Bonferroni post test using a confidence level of 0.05.

3.5 Results and Discussion

A series of substrates were prepared on SS316L including CH_3-P, CH_3-C, EG3-P, EG3-C and unmodified controls. All substrates were sterilized in a 24 well plate using ethanol and water then allowed to completely air dry before further use. The substrates were seeded randomly with fibroblast cells suspended in media at a density of 10,000 cells per well. The immersed substrates were incubated for twenty four hours at 37˚C

and 5% CO_2 and then stained using the Live/Dead Viability/Cytotoxicity components for thirty minutes under the same conditions. The attachment of live and dead cells to the control and modified SS316L substrates was examined using fluorescence microscopy at a 10X magnification. The acquired images revealed that the number of live cells was highest attached to the control. The number of cells on CH_3-P (Figure 13b) was the least in number followed by CH_3-C (Figure 13c), EG3-P (Figure 13d), and EG3-C (Figure 13e) respectively. The cells on the SAM-modified surfaces were rounded and small indicating a poor interaction with the surface while cells on the control were spread indicating a better interaction between the cells and the surface.

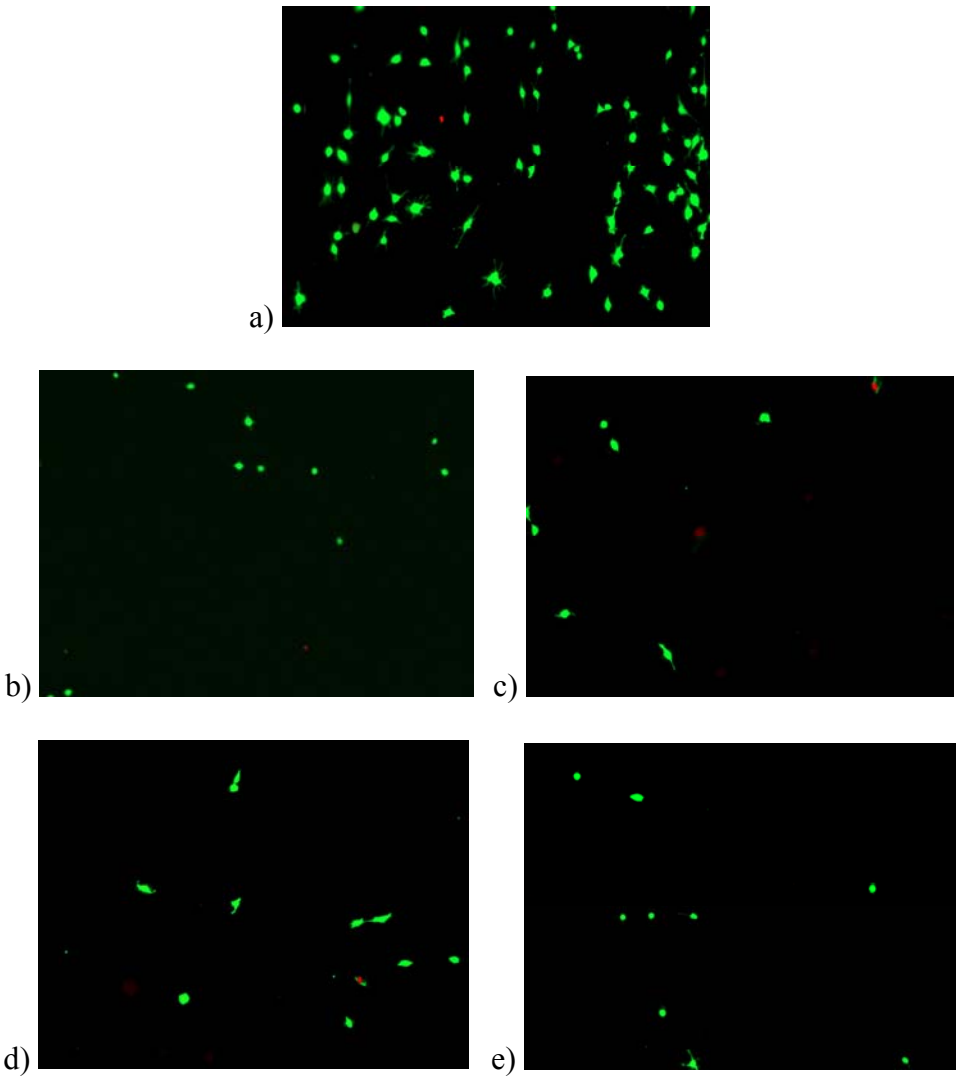


Figure 13: A sample of live/dead images using 10x magnification of a) Control b) CH_3-P c) CH_3-C d) EG3-P and e) EG3-C.

Viability of the cells attached to the surfaces was also determined by evaluating the fluorescent images. The numbers of dead cells (red) were very few when counted and compared to the live ones (green) on all of the surfaces. The viability on the control SS316L substrate and all of the modified substrates were greater than 90% as shown in

Table 2. This indicates that the cells attached to the control and modified substrates have a high viability, therefore, the substrates are not toxic to the cells.

Modification	Normalized Number of Live Cells	Standard Error (%)	Percentage Viability
Control	100	0	97
CH_3-P	22	6	92
CH_3-C	37	7	96
EG3-P	34	9	90
EG3-C	39	3	92

Table 2: Summary of results obtained from the viability/cytotoxicity experiments. The percentage viability was calculated by dividing the number of live cells by the total number of cells.

Statistical analysis was performed using one-way ANOVA followed by Bonferroni post-hoc test. The graph in Figure 14 compares the normalized number of cells on each of the modified substrates along with their statistical significance when compared to the SS316L unmodified control substrates. Comparison between independent experiments was enabled by normalizing the results to the control set at 100% attachment. There was a statistically significant difference between the number of cells attached to the control and all of the modified substrates ($p<0.001$) however the CH_3-P substrates provided the largest reduction in cellular attachment of 78% (standard error = 6%) versus the control; therefore, these substrates provided the most inert surfaces

when compared to the control. The EG3-P, CH_3-C and EG3-C surfaces also significantly reduced cellular attachment by 66% (standard error = 9%), 63% (standard error= 7%) and 61% (standard error= 3%) respectively versus the control, but not to the extent of the CH_3-P. The results are analogous to two studies that have shown that hydrophobic methyl terminated thiols on gold reduce cell attachment and spreading of fibroblast cells[56, 57] but in contrast to most thiols on gold studies.[25, 59-61]

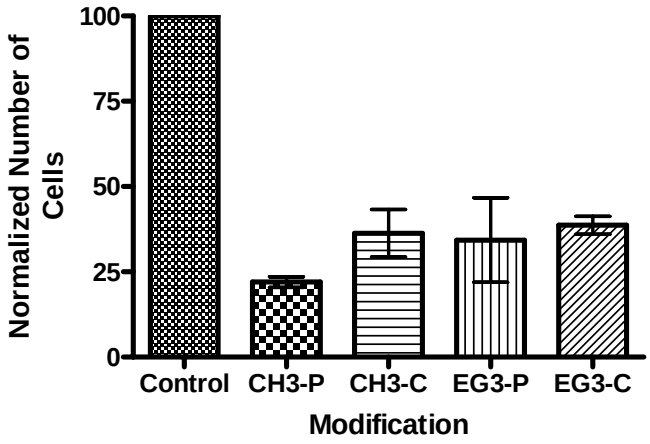


Figure 14: Normalized number of 3T3 cells on each modified SS316L substrate after 24 hours. $p<0.001$ for each modification compared to the control

Functional groups present at the surface/air interface must not be the only factor affecting the reduction in non-specific cell adhesion according to the results obtained from this research. This is concluded because while the CH_3-P provided the most inert surface, the CH_3-C did not reduce the adhesion of fibroblast cells to the extent of the CH_3-P substrates. A possible explanation is that the binding ability of the head group to the surface is also a factor in the ability of a surface to mitigate non-specific cell

attachment. Phosphonic acids have a higher pKa (pKa= 4) than carboxylic acids (pKa= 10) thus allowing for stronger binding of the molecule to the surface. This hypothesis is investigated by comparing the attachment of fibroblast cells to methyl and triethylene glycol-terminated molecules. Results show that the phosphonic acid substrates provide a larger reduction in cell attachment than the carboxylic acid substrates suggesting that stronger binding of the monolayer to the surface provides greater reduction of non-specific cell attachment.

3.6 Conclusions

There are many problems associated with the implantation of biomaterials such as vascular stents into the body including corrosion, non-specific protein and cell attachment, and infection. This work focused on eliminating the short- and long-term effects of non-specific protein and cell attachment to vascular biomaterials including restenosis and thrombosis by modifying the interfacial properties of stainless steel 316L with synthesized and commercially available methyl- and oligo(ethylene glycol)-terminated self-assembled monolayers. The modified substrates were incubated with 3T3 Swiss fibroblast cells for twenty four hours and evaluated for their ability to mitigate cell attachment.

Oligo(ethylene glycol)-terminated phosphonic and carboxylic acids were synthesized and analyzed using ^{1}HNMR spectroscopy, FTIR spectroscopy, and ESI MS. Results confirm the synthesis of 11-tri(ethylene glycol)undecylphosphonic acid, 12-tri(ethylene glycol)dodecylcarboxylic acid and 12-penta(ethylene glycol)dodecyl carboxylic acid.

The 11-tri(ethylene glycol)undecylphosphonic and 12-tri(ethylene glycol)dodecyl carboxylic acids were used along with octadecylphosphonic and octacosylcarboxylic acids to form self-assembled monolayers on the native oxide of stainless steel 316L. Monolayer formation was confirmed by DRIFT spectroscopy, contact angle goniometry, and AP MALDI-TOF MS. DRIFT spectroscopy was used to determine molecular ordering on the surface by analysis of the CH_2 antisymmetric and symmetric stretches of the spectra. Results confirmed that the monolayers were ordered on the surface except for the triethylene glycol-terminated phosphonic acid which may appear disordered due to the flexible nature of the triethylene glycol moiety. DRIFT spectroscopy also identified the binding of the molecules to the surface by analysis of the fingerprint region of the spectra. Analysis indicated bidentate binding of the phosphonic and carboxylic acids to the surface.

Contact angle goniometry was used to determine the wettability of unmodified and modified SS316L substrates. Methyl-terminated substrates had a much higher contact angle value when compared to the control surface indicating a hydrophobic interface. Triethylene glycol-terminated substrates had contact angle values that were just slightly higher than that of the control suggesting a mixture of hydrophobic and hydrophilic moieties at the interface potentially due to the flexible nature of the tail.

AP MALDI TOF MS was used to distinguish between monolayer and multilayer formation on the SS316L substrates by analyzing monomer and dimer regions of the spectra. Monomer peaks were present for octadecylphosphonic acid, 11-tri(ethylene glycol)undecylphosphonic acid and 12-tri(ethylene glycol)dodecylcarboxylic acid but dimer peaks were not present indicating monolayer formation on the substrates.

The ability of methyl- and tri(ethylene glycol)-terminated carboxylic and phosphonic acid SAMs on SS316L to resist the non-specific attachment of 3T3 fibroblast cells was also investigated. While the majority of studies on gold surfaces show triethylene glycol-terminated monolayers provided the most inert surfaces to fibroblast attachment, results obtained here indicate that octadecylphosphonic acid was best at reducing cellular attachment on SS316L followed by 11-tri(ethylene glycol)undecylphosphonic acid, octacosylcarboxylic acid and 12-tri(ethylene glycol) dodecylcarboxylic acid respectively. Therefore, the phosphonic acid modified substrates in general reduced non-specific fibroblast attachment more than their carboxylic acid counterparts. These results suggest the ability to resist non-specific cell attachment on SS316L is not only effected by the moiety present at the surface/air interface but also the binding strength of the acid to the surface. Overall, the results indicate that the ability of a surface to mitigate cell attachment is a complicated process that needs to be investigated further.

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